SUPPLEMENTAL INFORMATION

Neurotrophic factor MANF has a unique mechanism to rescue apoptotic neurons

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SUPPLEMANTAL EXPERIMENTAL PROCEDURES

Cloning, expression and purification of MANF–C-MANF corresponding to residues 96-158 of the maturated MANF was cloned into *Nco*I/*Xho*I restriction site of the pET15b vector (Novagen). Cloning to the *Nco*I site omits N-terminal His-Tag, otherwise included in the pET15b vector. ^{15}N- and/or ^{13}C-labelled full length MANF and C-MANF were expressed in OrigamiB(DE3) cells in order to obtain native protein with disulfide bonds. $^{15}NH_4Cl$ and ^{13}C-labelled glucose were used as sole sources of nitrogen and carbon, respectively. C-MANF was applied to ResourceS (GE Healthcare) with Buffer A (50 mM Na-PO_4, pH 6.5) and eluted with linear gradient mixed from Buffer A and Buffer B (50 mM Na-PO_4, pH 6.5, 1 M NaCl). Fractions containing C-MANF were pooled and concentrated with Vivaspin concentrator (2 kDa MWCO) to the final volume of 1 ml. Subsequently, sample was further purified with the Superdex30 16/60 gel filtration column (GE, Healthcare). Protein eluted at the volume of 60 ml, which is indicative of monomeric protein with molecular weight of ~7000 Da. NMR buffer was used in gel filtration. Fractions containing pure C-MANF were concentrated and stored at –80 °C until NMR data collection. All the purification steps were performed with the ÄKTA Purifier FLPC purification system (GE Healthcare). Cloning and purification of full-length MANF (residues 1-158) is described elsewhere (S1).

Cloning, expression and purification of C-Ku70 and Bcl-xL–In order to produce His-Tagged C-Ku70 and Bcl-xL, residues 558-609 of the human Ku70 and residues 1-209 of the human Bcl-xL were cloned into *Nde*I/*Xho*I site of the pET15b vector (Novagen). The construct of the C-Ku70 designed to exclude the nuclear localization signal, in order to keep the protein in cytoplasmic compartment when injected into SCG neurons. C-terminal hydrophobic α-helix of Bcl-xL protein was omitted from the cloned construct. Unlabelled Bcl-xL and C-Ku70 were expressed in BL21(DE3) using LB as growth media. Both proteins were purified by using Ni-NTA resin (Qiagen) according to manufacturer's instruction. Bcl-xL protein was further applied onto the CaptoQ anion chromatography (GE Healthcare). His-Tag was

removed from C-Ku70 protein by thrombin protease digestion and subsequently purified by Superdex30 16/60 gel filtration column (GE Healthcare). Proteins were concentrated and stored at –80 °C until used in cell cultivation experiments. All the purification steps were performed with the ÄKTA Purifier FLPC purification system (GE Healthcare).

NMR experiments for the assignment and structure calculations–NMR experiments were performed at 35 °C (FL-MANF) or at 25 ºC (C-MANF) on a Varian Unity INOVA 800 MHz spectrometer equipped with a 5 mm $\{^{15}N,^{13}C\}^{1}H$ triple-resonance x,y,z-gradient probehead, and a Varian Unity INOVA 600 MHz spectrometer, equipped either with a 5 mm $\{^{15}N,^{13}C\}^{1}H$ triple-resonance z-gradient coldprobe or $\{^{15}N,^{13}C\}^{1}H$ triple-resonance z-gradient probe. The assignment of main-chain resonances was carried out using a standard set of 3D triple-resonance experiments, i.e. HNCA/HN(CO)CA (full length MANF only), HNCACB/HN(CO)CACB and HN(CA)CO/HNCO experiment pairs were employed to establish intra-residual/sequential H(*i*)-N(*i*)-CA(*i*/*i*-1),CB(*i*/*i*-1) and H(*i*)-N(*i*)-CO(*i*/*i*-1) connectivities (S2). Owing to severe cross peak overlap, proline residues and exchange broadening of some amide resonances in full length MANF, additional Hα detected experiments HCAN and HCA(CO)N were employed for establishing ambiguous or missing sequential connectivities (S3).

Assignment of aliphatic side-chains was based on 3D H(CC)(CO)NH, (H)CC(CO)NH, HCCH-TOCSY and HCCH-COSY experiments (S2). Assignment was complemented with a set of novel HCCH-TOCSY experiments (Hellman *et al.*, unpublished results). Labile N-H resonances of Asn/Gln and His side-chain were assigned with assistance of a 3D NOESY-^{15}N-HSQC spectrum. Assignment of aromatic residues was carried out using a combination of 2D (HB)CB(CGCD)HD, (HB)CB(CGCDCE)HE, aromatic 3D HCCH-COSY spectra, and supplemented with the data from the ^{15}N-separated NOESY-HSQC experiment and a 3D ^{13}C-separated NOESY-HSQC experiment modified to simultaneously excite aliphatic and aromatic carbon resonances. Extent of the assignment has been described elsewhere (S4).

Interproton distance restraints were determined from the 3D ^{15}N-separated NOESY-HSQC, and a ^{13}C-separated NOESY-HSQC spectrum modified to simultaneously excite aliphatic and aromatic carbon resonances. The data were collected and processed using VNMR 6.1 revision C or VNMRJ 2.1 revision B software packages (Varian Inc, Palo Alto, CA). Visualization and spectrum analysis was performed using Sparky 3.106 (T.D. Goddard and D.G. Kneller, SPARKY 3, University of California, San Francisco, CA).

Structure calculation and analysis–The family of initial protein structures and automatically assigned NOE distance restraints were generated by using CYANA 2.0/NOEASSIGN structure calculation package (S5, S6). The 20 best out of 200 structures were selected, based on lowest target function values, for the simulated annealing with AMBER 8 (S7). The force field of Cornell (S8) was used with the generalized Born model (S9) to mimic water shell. Twenty ps simulated annealing protocol including 20000 time steps was performed using 18 Å non-bond cutoff. Fifteen conformers were selected, according to consideration of total AMBER energy and maximum distance and dihedral violations, to form the final ensemble of structures. The parameters characterizing the quality of the structures of full-length MANF and C-MANF are summarized in Table I and Table II, respectively. Structure based alignments and comparison of structures to homologous proteins of MANF's N-terminal and C-terminal domains were performed with the program Dali (S10). According to Ramachandran plot analyses, carried out with the program procheck-NMR (S11), 74.4%, 25.5% and 0.1% of the residues of full length MANF and 78.0%, 21.7% and 0.2% of the C-terminal domain of MANF were found in the most favourable, allowed and disallowed regions, respectively. The figures representing molecular structures were prepared with MolMol (S12).

Neuronal culture and microinjections–Cell cultivation and microinjection of the sympathetic neurons was performed as described earlier (S13). Plasmids for Ku70 and Bcl-xL have been described earlier (S13). Briefly, newborn mouse SCG neurons were grown with NGF (Promega) for 5–6 days then the nuclei were microinjected with the expression plasmids for FL-MANF, C-MANF, Ku70 or C-Ku70 together with a reporter

plasmid for enhanced green fluorescent protein (EGFP), using vector concentration of 10 ng/μl in each experiment. Similar results were achieved with plasmid concentrations of 50 ng/μl (not shown). For protein microinjection, recombinant FL-MANF, C-MANF, C-Ku70 or Bcl-xL proteins in PBS at 0.8 μg/μl were microinjected directly into the cytoplasm together with fluorescent reporter Dextran Texas Red (MW 70000 Da) (Invitrogen, Molecular Probes) that facilitates identification of the successfully injected neurons. The injected neurons were either deprived of NGF or treated with 30 μM etoposide (Sigma-Aldrich) or 200 nM staurosporine (Cell Signaling Technology). Living fluorescent (EGFP-expressing or Dextran Texas Red-containing) neurons were "blindly" counted three days later and expressed as percent of initial living fluorescent neurons counted 2–3 hours after microinjection. The experiments with plasmids were repeated on independent cultures 8 times for NGF deprivation and etoposide treatment, and 3 times for staurosporine treatment, whereas four independent protein injection experiments were performed. On average, 50–80 neurons were successfully injected per experimental group. The results were expressed as the mean ± the SEM. Data of each experimental group was compared with control plasmid pcDNA3 (vector) or PBS (in the protein injection experiments) by one-way ANOVA and *post hoc* Dunnett's *t* test. The null hypothesis was rejected at $p < 0.05$. For experiments with exogenous MANF, the neurons were grown with NGF for 5 days, then the cultures treated with etoposide (30 μg/ml) or washed to remove NGF and recombinant MANF (100 ng/ml) was added together with function-blocking anti-NGF antibodies. Also other doses of MANF (from 1 ng/ml to 1 μg/ml) did not promote survival of the neurons (not shown).

SUPPLEMENTAL TEXT

Structural homologues to the N-terminal domain of MANF. The structure is a reminiscent of 'closed leaf' fold, characterizing saposin-like proteins superfamily (S14, S15). Disulfide bond between C40 (helix α2) and C51 (helix α3) can readily be identified owing to several NOE cross-peaks observed between these residues. NOE connectivities can also be found between C6 (helix α1) and C93 (3_{10} helix). In our solution structure C9 (helix α1) and C82 (helix α5) are spatially in close proximity and are likely to form disulfide bridge. C-terminal domain contains two additional cysteines (*vide infra*). According to the structural alignment with the program Dali (S10), similarities to structures of NK-lysin (S16) (PDB: 1nkl; Z-score 6.5, RMSD 2.7 Å), granulysin (S17) (PDB: 1l9l; Z-score 4.2, RMSD 2.9 Å), saposin D (S18) (PDB: 3bqq; Z-score 5.6, RMSD 2.9 Å), and saposin C (S19) (PDB: 1m12; Z-score 3.6, RMSD 3.5 Å) can be found. These homologues are members of saposin protein superfamily, all comprising ca. 80 residues, which is named after small lysosomal protein, saposin. Despite their low sequence identity, all saposin-like proteins (SAPLIPs) share characteristic fold, a 'closed leaf' topology characterized by five α-helices connected with three conserved disulphide bonds. Apparently, three disulfide bridges located in the N-terminal part of MANF have structural characteristics similar to proteins belonging to saposin superfamily.

Although functions of saposins span from cofactors of enzymes in lipid metabolism to antimicrobial effector molecules in T cells, they all are lipid and membrane binding proteins. SAPLIPs are known to anchor to membrane where they can disturb well-packed lipid order or even isolate lipid molecules to be further degraded by soluble enzymes. While many saposins are active in monomeric form, membrane or lipid binding can also induce conformational change and oligomerization. Membrane permeabilization proteins form pores to membrane, such as antimicrobial NK-lysin and granulysin. Those proteins have several positively charged residues clustered to the helix 3 and 2, which make the first contact with the membrane leading to electroporation. SAPLIPs involved in unpacking lipid molecules from membranes lack positively charged cluster but are typically negatively charged (S14).

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Figure S1. Stereo images representing overall fold of MANF. The main chain representation of the 15 lowest energy superimposed structures. Structures are superimposed according to *A*) residues 7-91 belonging to N-terminal domain of MANF, *B*) residues 112-147 belonging to C-terminal domain of MANF, and *D*) isolated C-terminal domain of MANF. Stereo images of the ribbon presentations of *C*) full-length MANF and *E*) C-MANF.

Fig. S1.